SYSTEM AND METHOD FOR CARRYING OUT MULTIPLE BINDING REACTIONS IN AN ARRAY FORMAT

FIELD OF THE INVENTION

This invention relates to methods for carrying out multiple binding reactions between bio-molecules in an array-format and more specifically to such systems and methods using biosensors and more specifically using optical

5 detection methods such as surface plasmon resonance (SPR).

BACKGROUND OF THE INVENTION

In the new era of genomics, proteomics and bio-informatics, a vast number of proteins, new drug targets and small molecules are being investigated intensively and in

10 high-throughput fashion. Although the full mapping of the human genome is done, genomics cannot provide a complete understanding of cellular processes which involve functional interactions between proteins and other molecules as well. Therefore, proteomics may be considered as a cutting-edge area of research today, bridging genomics and cell function.

15 Current technological methods for analyzing a large number of functional interactions between bio-molecules (especially proteins) include well-plate based screening systems (e.g., ELISA), cell-based assays, soluble reactants screening (e.g., radio immunoassays) and solid-phase assays (e.g., DNA-chips). Today, there is an obvious lack of high throughput technology which enables real-time, label-free

20 monitoring of kinetics of multiple bio-molecular interactions (especially proteins).

The major current limitation in developing such solid-phase based-assays stems from the complexity and variability of proteins. Proteins, in contrast to DNA molecules which are used in producing DNA-chips, are less stable, and generally must kept

hydrated and in an active structure and conformation. Also, proteins are very sensitive to chemical and physical changes (e.g., temperature). Finally, with regard to solid-phase kinetic studies, the amount or capacity of an immobilized protein must be known in order to perform an accurate, full kinetic study.

5   As used herein, the termm *"biosensor"* refers to combination of a receptor . surface for molecular recognition and a transducer for generating signals indicative of binding to the surface.

Various related optical methods can be used to measure kinetic binding interactions between bio-molecules. These include, among others, Surface

10   Plasmon Resonance (SPR), total internal reflection fluorescence (TIRF) and evanescent wave elipsometry. It is known in the art to use biosensors and mainly SPR for such purpose. A kinetic binding reaction involves a first molecular species referred to herein as *"the probe"*. The probe is adsorbed to the sensor surface, and a solution containing a second molecular species, referred to herein

15   as *"the target"* is then allowed to flow over the probe molecules adsorbed onto the sensor surface. As is known in the art and in commercially available devices, a standard kinetic binding interaction measurement can be described by the following procedure:

(1) Chemical activation of solid-phase surface with a chemical activator

20   (e.g., EDC/NHS); (2) Immobilization of a 'probe' molecule on a
           chemically-activated surface; (3) Washing and blocking of un-occupied
           activated groups with a blocker such as 1M ethanolamine; (4) Addition of
           one concentration of a 'target' molecule; (5) Washing and regeneration of
           the 'probe' with appropriate regenerating chemicals (e.g., 50 mM NaOH,

25   0.05% SDS); (6) Addition of another concentration of 'target'; (7)
           Repeat stages 4-6, at least five times, each time with a different 'target'
           concentration.

In one aspect of this invention, the invention provides a method, referred to herein as *"One-Shot Kinetics"* (OSK). for obtaining one or more kinetic

30   parameters of a binding reaction As shown below, this method allows carrying

out a plurality of binding reactions without the need of the regeneration stage which is known to be harmful to the 'probe'.

In general, any binding event between probe and target molecules can alter an SPR detection parameter which is than is used to monitor the binding 5   reaction. The change in the detection parameter over time is used to determine a characteristic of the binding reaction, such as an association or dissociation constant rates as well as affinity. It is known to use surface Plasmon resonance (SPR) as the method of detection. SPR devices and methods are very sensitive to changes in an optical property of a probe layer and have proven to be useful in 10   detecting changes in an optical property of a probe layer generated by relatively small stimuli.

An SPR probe layer may be configured as a multi-analyte "microarray" in which at each of a plurality of discrete regions, "microspots" on the sensor surface a probe material for interaction with a target material is adsorbed. Berger 15   *et al.,* describes a method for preparing a probe array and for presenting targets to the probe array so as to monitor the binding of the targets to the probes ("Surface Plasmon Resonance Multi-sensing", Anal. Chem. Vol. 70, February 1998, pp 703-706,.

PCT publication WO 02/055993, discloses the use of electrostatic fields 20   and chemical cross-linking for binding probes to a sensor surface.

SUMMARY OF THE INVENTION

The present invention provides a system and method for determining kinetic parameters of one or more binding reactions between one or more probes and one or more targets. The probes and targets may be, for example, peptides, proteins, 25   nucleic acids or polysaccharides. The probes and targets may be of the same species. For example, both of them may be proteins. Alternatively, the probes and targets may be of different species. For example, the probes may be nucleic acids, while the targets are proteins.

The system of the invention uses any detection method suitable for use in biosensors. More specifically, it uses a detection method based on an evanescent wave phenomenon such as surface plasmon resonance (SPR), critical angle refractometry, total internal reflection fluorescence (TIRF), total internal reflection phosphorescence, total internal reflection light scattering, evanescent wave elipsometry or Brewster angle reflectometry. The detection method makes use of a surface that allows a plurality of binding reactions to be monitored simultaneously. The method comprises adsorbing the probes to the sensor surface at different locations on the surface, for example by means of micro-fluidic methods using a chemical surface activator, or using a localized electric field. Each target is then presented to its respective probe adsorbed to the surface. The binding reactions between each pair of probe and target are monitored simultaneously.

In its first aspect, the present invention provides a system and method for determination of the kinetic parameters of a binding reaction, referred to herein as *"One-Shot Kinetics"* (OSK). This method allows carrying out a plurality of binding reactions without the need of the regeneration stage and without the need of repeated experiments which is known to be harmful to the 'probe'.. In this preferred embodiment of the method of the invention, a single probe species is adsorbed to microspots on a surface such as an SPR surface under a plurality of conditions, for example at different concentrations or pH, in order to obtain different probe densities. Some conditions may be repeated in order to obtain density duplicates. A single target species is then presented to the microspots at a plurality of concentrations. A plurality of probe density and target concentration combinations is thus obtained. The pluralities of reactions are monitored simultaneously and signals indicative of the binding reactions are obtained and analyzed so as to produce a kinetic analysis of the binding. The kinetic analysis may comprise of, for example, calculating an association constant or a dissociation constant or affinity constant for the binding of the probe to the target.

In its second aspect, the invention provides a method, referred to herein as *"array-screening"*, for simultaneously monitoring a plurality of binding reactions

between a plurality of probes and one or more targets so as to obtain analysis of many binding reactions. In one embodiment of this aspect of the invention, a specific probe species is adsorbed to the surface at different one of a plurality of microspots so that each probe in each microspot may be selected independently of the probes on the other microspots. A target species is then presented to the probe in each microspot. Binding of the targets to the probes in the plurality of microspots is monitored simultaneously and signals indicative of the binding reactions are analyzed so as to produce analysis of the binding. The analysis may comprise of, for example, determining the existence of a detectable interaction at each microspot or calculating an equilibrium constant for the binding of the probe to the target at each microspot or determining the kinetics of binding.

The probes may be localized at different locations on the surface, for example, by means of micro-fluidic methods. The location on the surface may be activated, for example by using a chemical activator, or by applying an electric field, or by exposure to light (photo-activation). In order to achieve, localization, it is known to form a chemical thin layer covering a specific region of the surface, frequently referred as a binding layer. The binding layer may include different functional groups that are chemically activated, either by contact with chemical reagents, by applying an electric field, or by exposure to light (photo-activation).

Activation by an electric field may be carried out in two principal ways: (A) inducing an electrochemical reaction (reduction or oxidation) of functional groups in the binding layer. (B) applying an electric field so as to attaract charged bio-molecules to the surface, and thus enhance the immobilization reaction; thus forming a higher local concentration of the probe molecules at the surface.

The most common binding layers for protein immobilization contain carboxylic groups. These carboxylic groups are activated by exposing the surface to accepted chemical activators, generally a mixture of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and NHS (N-hydroxysuccinimide)) in an aqueous solution. As a result, active NHS esters are formed. When the activated surface is contacted with a protein solution, the NHS esters react efficiently with

nucleophilic groups on the protein backbone, mainly with amino groups to form stable amide bonds. Thus, covalent immobilization of proteins is achieved. Other methods for chemical activation include attachment of a molecule that exhibits a high affinity to the candidate for immobilization, e.g. attachment of avidin or an

5 avidin derivative for immobilization of biotin-labeled molecules.

The invention also provides a method for preparing a probe array for use in the method of the invention for monitoring binding reactions.

Thus, in its first aspect, the invention provides a method for determining one or more kinetic parameters of binding between a first binding member and a second

10 binding member comprising:

    (a)   adsorbing the first binding member to a surface at a plurality of microspots;

    (b)   presenting the second binding member to the first binding member at each of the microspots, there being a plurality of

15   combinations of first binding member surface density and second binding member concentration among the plurality of microspots;

    (c)   simultaneously obtaining data indicative of a binding reaction between the first and second binding members at each of the

20   plurality of microspots by a biosensor detection method; and

    (d)   processing the data so as to obtain one or more kinetic parameters of binding between the first and second binding members.

In its second aspect, the invention provides a method for localizing a

25 molecular species at each of two or more microspots on a surface, comprising, for each of one or more localization regions:

    (a)   activating the surface in the localization region;

    (b)   for each of one or more microspots in the localization region, adsorbing a molecular species to the microspot; and

30   **(c)**   optionally deactivating the localization region.

In its third aspect, the invention provides a probe array produced by the method of the invention.

In its fourth aspect the invention provides a system for simultaneously monitoring a plurality of binding reactions between one or more probe species and one or more

5   target species comprising

   (a)   A surface;

   (b)   An applicator capable of applying probe species to microspots on the surface so as to allow the probe species to be adsorbed to the microspot, the applicator being further capable of presenting a target

10   to each probe species adsorbed to the surface;

   (c)   A photosurface receiving light reflected from the surface and generating signals indicative of the binding of the targets to the probes; and

   (d)   A processor configured to receive the signals generated by the

15   photosurface and to analyze the signals so as to produce a kinetic analysis of the binding.

BRIEF DESCRIPTION OF FIGURES

   In order to understand the invention and to see how it may be carried out

20 in practice, a preferred embodiment will now be described by way of non-limiting example only, with reference to the following accompanying drawings, in which:

   Fig. 1 shows a system for performing multiple binding reactions in accordance with one embodiment of the invention;

25   **Fig. 2** shows a system for performing multiple binding reactions in accordance with another embodiment of the invention;

   Fig. 3 shows a method for preparing a probe array in accordance with one embodiment of the invention;

Fig. 4 shows a method for preparing a probe array in accordance with another embodiment of the invention;

Fig. 5 shows binding curves of IL-4 to anti-IL-4 antibody obtained by the method of the invention; and

5   Fig. 6 shows binding curves of five antigen targets to six antibody probes.

Fig. 7 shows binding curves of various compound targets to six CYP450 enzyme probes.

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

Figs. 1a and 1b schematically show a system **10** for simultaneously
10 carrying out multiple binding reactions in accordance with one embodiment of this aspect of the invention. The system **10** includes an SPR device **80** comprising an array **24** of light sources **26** and a prism **30** having a sensor surface **32**. The light sources **26** provide light at a wavelength appropriate for SPR applications as is known in the art. The light array **24** is positioned at the focal
15 plane of an optical system schematically represented by a lens **46** having an optical axis **48**. Lens **46** collects and collimates light from each light source **26** into a beam of parallel light rays and directs the collimated light so that it is incident on an "input" prism surface **50** of prism **30**. Light directed by collimator **46** that is incident on input surface **50** enters prism **30** and is incident on sensor
20 surface **32**.

All light incidents on the sensor surface **32** from a given light source **26** is incident on the sensor surface at substantially a same incident angle and light from different light sources **26** is incident on the sensor surface at different incident angles. The angle at which light from a given light source **26** is incident
25 on sensor **26** on sensor surface **32** is determined by the position of the given light source along the axis of the array **24**, the focal length of the lens **46** and the index of refraction of the material from which prism **30** is formed. The SPR device **80** may include a *"displacement plate"* (not shown) formed from a transparent material that is positioned between light source array **24** and prism **30**. The

angular orientation of displacement plate is set so that the normal to the displacement plate is oriented at a desired angle with respect to the optic axis **48**.

Light incident on sensor surface **32** that is reflected from the surface exits prism **30** through an output prism surface **52** and is collected and imaged by a
5 camera **55** having a lens **53** and a two dimensional photosurface **54** such as a CCD. A polarizer (not shown) is positioned between the array **24** and the prism **30** or preferably between the prism **30** and the camera **55**. The polarizer linearly polarizes light received by photosurface **54** so that relative to sensor surface **32** it has substantially only a p component of polarization.

10 The camera **55** outputs signals **57** that are indicative of images formed on the photosurface **54**. The signals **57** are input to a processor **59** having a memory **63** for storing signals **57**. The processor **59** is configured to analyze the signals as described below. Any of the signals **57** or results of the analysis performed by the processor may be displayed on an associated display screen **65**.

15 The system **10** includes a flow cell **34** having m microchannels **36** for flowing liquid across and in contact with the sensor surface **32**. In the device **80**, m=5 microchannels **36a** to **36e** are shown. This is by way of example only, and the method of the invention may be carried out using flow cell having any number m of microchannels. The outer form of flow cell **34** is shown in ghost
20 lines and details of internal features, such as microchannels **36**, of the flow cell are shown in solid lines for clarity of presentation. Each microchannel **36** has at one end an inlet (not visible in the perspectives shown in Figs. 1a and 1b) and, at its other end, an outlet **61** through which fluid flowing in the microchannel exits the microchannel. Each of the inlets is adapted to be independently connected to
25 a suitable pumping apparatus (not shown) in order to introduce a fluid independently into each of the m microchannels **36**.

In the system **10**, the flow cell **34** is mountable onto the SPR surface in two orientations. One of the two orientations is shown in Fig. 1a and is referred to herein as *"the probe orientation"*. The second orientation, shown in Fig. 1b is
30 referred to herein as the *"target orientation"*. In each of the two orientations, the

microchannels are perpendicular to the microchannels in the other orientation. Each microchannel **36** is open on a side of the microchannel facing sensor surface **32** so that fluid flowing in the microchannel, in either orientation, contacts the SPR surface in a rectangular region. In the probe orientation shown

5 in Fig. 1a, fluid flowing in a microchannel contacts the sensor surface at a respective rectangular region **42** referred to herein as the microchannel's *"probe region"* (see Fig. 1b). In the target orientation shown in Fig. 1b, fluid flowing in a microchannel contacts the sensor surface at a respective rectangular region **43** referred to herein as the microchannel's *"target region"* (see Fig. 1a). The probe

10 regions and the target regions are thus perpendicular to each other. Regions of some microchannels **36** in the system **10** in Figs. 1a and 1b are cut away to show microspots **58** formed at the crossover regions of the probe regions and the target regions.

Fig. 2 schematically shows a system **11** for simultaneously carrying out

15 multiple binding assays in accordance with another embodiment of this aspect of the invention. The system **11** includes an SPR device **20** having several components in common with the SPR device **80** shown in Fig. 1, and similar components are indicated with the same reference numeral in both figures. In particular, the SPR device **80** includes an optical system comprising an array **24**

20 of light sources **26**, a prism **30** having a sensor surface **32**, a lens **46** having an optical axis **48**, and a two dimensional photosurface **54** such as a CCD. A suitable SPR conductor (not shown) is formed on the sensor surface.

The system **11** includes a flow cell **34** having m microchannels **36** for flowing liquid across and in contact with the sensor surface **32**. In the device **80**,

25 m=5 microchannels **36a** to **36e** are shown. This is by way of example only, and the method of the invention may be carried out using a flow cell having any number m of microchannels. The outer form of flow cell **34** is shown in ghost lines and details of internal features, such as microchannels **36**, of the flow cell are shown in solid lines for clarity of presentation. Each microchannel **36** has at

30 one end an inlet (not visible in the perspectives shown in Figs. 1a and 1b) and, at

its other end, an outlet 61 through which fluid flowing in the microchannel exits the microchannel. Each of the inlets is adapted to be independently connected to a suitable pumping apparatus (not shown) in order to introduce a fluid independently into each of the m microchannels 36.

5   The SPR device 20 has n strip electrodes 33. The n strip electrodes are used to create n independently activatable regions. While n=5 strip electrodes 33a to 33b are shown in Fig. 2, this is by way of example only and the method of the invention may be carried out with an SPR device having any number of strip electrodes.

10   In the system 11, the flow cell 34 is mounted onto prism 30 so that the m microchannels are perpendicular to the n strip-electrodes 33. Each microchannel 36 is open on a side of the microchannel facing sensor surface 32 so that fluid flowing in the microchannel contacts each strip electrode 33 at a microspot 58 located at the crossover region of the microchannel with the strip electrode. In an

15 SPR device having m microchannels and n strip electrodes, a total of m X n microspots are formed at eh crossover regions of the m microchannels with the n strip electrodes. Regions of some microchannels 36 in SPR device 20 in Fig. 2 are cut away to show microspots 58.

          Each strip electrode 33 is independently connected to a power supply 60.

20 Power supply 60 is controllable to independently bring each strip electrode 33 to a voltage relative to a reference electrode 62 connected to the power supply so as to generate an electric field having a component perpendicular to the sensor surface 32. The electric field passes through the lumen of the microchannels 36 at the crossover region of the microchannels with the strip electrode.

25   Fig. 3 schematically shows a method for preparing a probe array on a surface 70 in accordance with one embodiment of the method of the invention. In Fig. 3a, a first surface region 72a on the surface 70 is activated. Activation of a surface region allows probe molecules to be adsorbed to the surface region. One or more probe species 71 are then adsorbed to the activated first surface region

30 72 (Fig. 3b) at distinct microspots in the first surface region 72. Fig. 3b

schematically shows the application of 6 probe species **71a** to **71f** to the activated first surface region **72a**. This is by way of example only and the method of the invention may be carried out with any number of probe species **71** being adsorbed to the first surface region **72**. This produces the probe array shown in

5   Fig. 3c, in which each probe species is adsorbed to a different microspot **74**. Fig. 3c shows 6 microspots **74a** to **74f**. The probe species may all be different or some of the probe species may be the same possibly at different concentrations.

The first surface region is now deactivated and a second surface region **72b** is activated. One or more probe species are then adsorbed to distinct

10   microspots on the second surface region **72b**, as explained above for the first surface region **72a**. The process is repeated, each time activating a different one of the surface regions **72** until probe species have been adsorbed to microspots on each of the surface regions **72**. This produces the probe array shown in Fig. 3d in which a plurality of probe species is adsorbed to microspots **75**. In Fig. 3d, 6

15   surface regions **72a** to **72f** are shown. This is by way of example only, and the method of the invention may be carried out with any number of surface regions **72**. In the example shown in Fig. 3d, on each of the 6 surface regions **72**, 6 probe species were adsorbed. This produces the array of 36 microspots shown in Fig. 3d. The probe species adsorbed on different surface regions may be different, so

20   that up to 36 different probe species may be adsorbed onto the surface **70**.

After the probe array on the surface **70** has been prepared, for each surface region, a target species may presented to the probe species adsorbed to the microspots.

The method of preparing a probe array on a surface shown in Fig. 3 will

25   now be demonstrated with reference to the system **10** of Fig. 1. In this example, m^2 probe species are to be adsorbed to the SPR surface at the m^2 microspots **58** (m^2=25 in the SPR device **80** of Fig. 1) located at the m^2 crossover regions of the m probe regions with the m target regions. To prepare an appropriate microarray of the m^2 probes on the probe regions, the flow cell **34** is first placed in one

30   orientation (Fig. 1b) and buffer or water is first pumped through the first

microchannels 36 in order to clean and prepare the first surface region 43a. Flow of buffer or water through the first microchannel 36a is then stopped a solution of a chemical surface activator is then made to flow through the first microchannel 36a in order to activate the first surface region 43a. The first

5   surface region is now activated.

The flow cell is now rotated 90° to bring it from the target orientation shown in Fig. 1b to the probe orientation shown in Fig. 1a. An appropriate solution comprising a probe species is pumped through each of the m microchannels 36. The m probe species may all be different, or some may be the

10 same probe species, possibly at different concentrations. As a result of the activation of the first surface region 43a, each probe species is adsorbed to the first surface region 43a and is not adsorbed by the other m-1 surface regions 43b-43e. Each of the probe species is thereby immobilized at a different one of the m microspots 58 located at the m crossover regions of the m probe regions 42 with

15 the first surface region 54a. The probes are substantially prevented from immobilizing at the m X (m-1) microspots 58 located at the crossover regions of the m probe regions 42 with the m-1 other surface regions 43b-43e.

During immobilization of the probes, the process of immobilization and the quantities of probe proteins immobilized at each microspot 58 are monitored

20 by performing an SPR angular scan of the sensor surface 64, as is known in the art. The signals 57 generated by the CCD 54 responsive to light from each light source 26 reflected at each microspot 58 on the first surface region 43a during adsorption of the probes are input to the processor 59. The processor 59 is configured to analyze the signals so as to determine an SPR parameter for the

25 microspot. The SPR parameter may be, for example, the SPR resonance angle, resonance wavelength, or the reflectance and phase changes that characterize a surface Plasmon resonance. The processor is further configured to analyze the SPR parameter so as to monitor accumulation of the probe immobilized at the microspot. Signals 57 from microspots of the other m-1 surface regions and

30 from regions of the probe surface that are not crossover regions are analyzed by

the processor to correct and normalize signals from crossover regions of the first surface region.

After termination of the flow of the probe solutions in the microchannels, the flow cell is rotated 90° back to the target orientation (Fig. 1b) and a solution
5   containing a surface activator blocker is made to flow through the microchannels 36 to prevent further binding to the first surface region.

The above-described process is repeated for each of the other remaining m-1 surface regions 43b -43a with m probe solutions, until a probe species has been immobilized at each of as many as m^2 different microspots 58 located at the
10 m^2 crossover regions of the m probe regions and the m surface regions. Each surface region 43 may thus be activated individually. As used herein, the term "*activatable region*" is used to refer to a region that can, when activated, bind one or more probe species. Thus, with the method of the invention, a probe microarray comprising as many as m^2 different probe species may be formed on
15 the SPR surface of the SPR device 80.

Following preparation of the probe microarray, a solution containing a target species is made to flow in each of the m microchannels 36 in the flow cell with the flow cell in the target orientation. The m target species may all be different, or some of the target species may be the same, possibly at different
20 concentrations. Thus, for each of the m target solutions, the target is presented to each of the m probe species in the m microspots 58 located at the m crossover regions of the target's target region with the m probe regions. The signals 57 provided by the CCD 54 responsive to light from the light sources reflected from each of the m^2 microspots 58 during flow of the target molecules in the
25 microchannels are input to the processor 59. The processor 59 is configured to analyze these signals in order to monitor the binding of target to probe at each microspot. A total of as many as m^2 binding reactions can thus be monitored simultaneously involving as many as m^2 different probe species and as many as m different target species. As known in the art, reference surface must be used
30 and be subtracted from any signal obtained from 'active spot'. In one aspect of

this invention, and as a novel outcome of the method, the surface between the
spots, termed "inter-spot" is used as a reference surface.

The method of preparing a probe array on a surface shown in Fig. 3 will
now be demonstrated with reference to the system 11 of Fig. 2. In this example,
5 m X n probe species are to be adsorbed to the SPR surface that at the m X n
microspots 58 (m X n = 25 in the system 11 of Fig. 2) located at the m X n
crossover regions of the m microchannels 36 with the n strip electrodes 33. To
prepare an appropriate microarray of the m X n probes on the strip electrodes 33,
buffer or water is first pumped through the microchannels 36 to clean and
10 prepare the strip electrodes for immobilization of the probe molecules at the
microspots 58. Flow of buffer or water through the m microchannels is then
stopped and the first strip electrode 33a is now activated as explained above.
The remaining strip electrodes are all brought to a potential with respect to the
reference electrode 62 having a polarity opposite to that of the first electrode. An
15 appropriate solution comprising a probe species is pumped through each of the m
microchannels 36. The m probe species may all be different, or some may be the
same probe species, possibly at different concentrations. As a result of the
activation of the first strip electrode 33a and the charge on the m probe species in
the microchannels, each probe species is adsorbed to the first strip electrode 33a
20 and is not adsorbed by the other n-1 strip electrodes 33b-33e. Each of the probe
species is thereby immobilized at a different one of the m microspots 58 located
at the m crossover regions of the m microchannels with the first strip electrode
33a. The probes are substantially prevented from immobilizing at the m X (n-1)
microspots 58 located at the crossover regions of the m microchannels with the
25 n-1 other strip electrodes 33b-33e. .

During immobilization of the probes, the process of immobilization and
the quantities of probe proteins immobilized at each microspot 58 are monitored
by performing an SPR angular scan of the sensor surface 64, as is known in the
art. The signals 57 generated by the CCD 54 responsive to light from each light
30 source 26 reflected at each microspot 58 on the first strip electrode 33a during

adsorption of the probes are input to the processor **59**. The processor **59** is configured to analyze the signals so as to determine an SPR parameter for the microspot. The processor is further configured to analyze the SPR parameter so as to monitor accumulation of the probe immobilized at the microspot. Signals
5   **57** from microspots of the other n-1 strip electrodes **33b-33e** and from regions of the probe surface that are not crossover regions are analyzed by the processor to correct and normalize signals from crossover regions of the first target region.

After termination of the flow of the probe solutions in the microchannels, buffer or water is again made to flow through the microchannels **36** to eliminate
10   unbound probe proteins.

The above-described process is repeated for each of the other remaining n-1 strip electrodes **33b-33e** with m probe solutions, until a probe species has been immobilized at each of as many as m X n different microspots **58** located at the m X n crossover regions of the m microchannels **36** and the n strip electrodes **33**.
15   Thus, with the method of the invention, a probe microarray comprising as many as m X n different probe species may be formed on the SPR surface of the SPR device **20**.

Following preparation of the probe microarray, a solution containing a target species is made to flow in each of the m microchannels **36**. The m target
20   species may all be different, or some of the target species may be the same, possibly at different concentrations. Thus, for each of the m target solutions, the target is presented to each of the n probe species in the n microspots **58** located at the n crossover regions of the target's microchannel with the n strip electrodes. The signals **57** provided by the CCD **54** responsive to light from the light sources
25   reflected from each of the m^2 microspots **58** during flow of the target molecules in the microchannels are input to the processor **59**. The processor **59** is configured to analyze these signals in order to monitor the binding of target to probe at each microspot. A total of as many as m X n binding reactions can thus be monitored simultaneously involving as many as m X n different probe species
30   and as many as m different target species. Ina preferred embodiment, a region of

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the surface, referred to as "an interspot" is used as a reference surface to provide a reference signal.

Fig. 4 shows a method for preparing a probe array on a surface **80** in accordance with another embodiment of the method of the invention (termed

5 "OSK" or "one-shot kinetics"). This embodiment may be used when it is desired to perform a binding assay involving one probe species and one target species at different combinations of probe and target concentrations. In this embodiment, m probe regions **82** are simultaneously activated. 6 probe regions **82a** to **82f** are shown in Fig. 4a. This is by way of example only, and the method may be carried

10 out with any number of probe regions. The m probe regions **82** are activated and the probe is adsorbed onto the probe regions **82**. A different probe concentration is adsorbed onto each probe region. One of the probe regions **82f** may be used as a reference region upon which no probe is adsorbed.

As depicted in Fig. 4b, n concentrations of the target are then presented to

15 the probe array (designated analytes 1-6). Each concentration is presented to a different microspot on each of the probe regions. The reaction assay thus consists of all of the m X n combinations of the probe and target concentrations.

The method of performing a binding assay shown in Fig. 4 will now be demonstrated with reference to the system 10 of Fig. 1. This embodiment is used

20 when it is desired to perform a binding assay involving one probe species and one target species at different combinations of probe and target concentrations. The probe species is applied to each of the m probe regions at a different concentration, and the target is applied to each of the m target regions at a different concentration. To prepare this microarray, the flow cell **34** is first

25 placed in the probe orientation (Fig. 1a) and buffer or water is first pumped through the m microchannels **36** in order to clean and prepare the m probe regions **42**. Flow of buffer or water through the m microchannels **36** is then stopped and any residual buffer or water in the flow system is drained away. A solution of a chemical surface activator is then made to flow through the m

30 microchannels **36** in order to activate the m probe regions **42**. The surface

activator may be, for example, EDC/NHS. The m probe regions are now activated.

An appropriate solution comprising the probe is pumped through each of the m microchannels **36**. In this embodiment, the probe is present in each of the

5   different microchannels at a different concentration. As a result of the activation of the target regions **42**, probe molecules in each microchannel are adsorbed to the n microspots **58** in contact with the microchannel.

During immobilization of the probes, the process of immobilization and the quantities of probe proteins immobilized at each microspot **58** are monitored

10 by performing an SPR angular scan of the sensor surface **64**, as is known in the art. The signals **57** generated by the CCD **54** responsive to light from each light source **26** reflected at each probe region **42** during adsorption of the probe are input to the processor **59**. The processor **59** is configured to analyze the signals so as to determine an SPR parameter for each probe region **42**. The processor is

15 further configured to analyze the SPR parameter so as to monitor accumulation of the probe immobilized on each probe region **42**. Signals **57** regions of the SPR surface not in a probe region are analyzed by the processor to correct and normalize signals from the probe regions.

After termination of the flow of the probe solutions in the microchannels,

20 a solution containing a surface activator blocker is made to flow through the microchannels **36** to prevent further binding of molecules to the SPR surface. The surface activator blocker may be, for example, ethanolamine.

The flow cell is now rotated 90° from the probe orientation to the target orientation (Fig. 1b). A solution containing the target species is made to flow in

25 each of the m microchannels **36** of the flow cell. In this embodiment, the target is present in the different microchannels at a different concentration. Thus, for each of the m target solutions, the target is presented to each of the m probe concentrations species in the m microspots **58** located at the m crossover regions of the target solution with the m probe regions. The signals **57** provided by the

30 CCD **54** responsive to light from the light sources reflected from each of the m^2

microspots **58** during flow of the target molecules in the microchannels are input to the processor **59**. The processor **59** is configured to analyze these signals in order to monitor the binding of target to probe at each microspot. In this embodiment, a total of m^2 binding reactions are thus monitored simultaneously

5 involving as many as m^2 different combinations of probe concentration and target concentration. This allows the collection of kinetic data on the binding of the target to the probe for kinetic analysis in a single binding assay, without the need to regenerate the surface at any time. This is in contrast to prior art methods in which reactions are performed sequentially, each time with a different

10 combination of probe and target concentrations which requires regeneration of the surface between successive binding reactions.

The method of performing a binding assay shown in Fig. 4 will now be demonstrated with reference to the system **11** of Fig. 2. The probe species is applied to each of the m probe regions at a different concentration, and the target

15 is applied to each of the m target regions at a different concentration. To prepare this microarray, the flow cell **34** is positioned as shown in Fig. 2 with the m microchannels **36** perpendicular to the n strip electrodes **33**. Buffer or water is first pumped through the microchannels **36** to clean and prepare the SPR surface in contact with the microchannels Flow of buffer or water through the m

20 microchannels is then stopped and the n strip electrodes **33** are now activated as explained above. An appropriate solution comprising the probe is pumped through each of the m microchannels **36**. In this embodiment, the probe is present in each of the different microchannels at a different concentration. As a result of the activation of the strip electrodes **33** and the charge on the probe in the

25 microchannels, probe molecules are adsorbed to the strip electrodes **33**. Probe molecules are thereby immobilized at a different one of the n microspots **58** located at the n crossover regions of the microchannel with the n strip electrodes **33**.

During immobilization of the probes, the process of immobilization and

30 the quantities of probe proteins immobilized at each microspot **58** are monitored

by performing an SPR angular scan of the sensor surface **64**, as is known in the art. The signals **57** generated by the CCD **54** responsive to light from each light source **26** reflected at each microspot **58** on the first strip electrode **33a** during adsorption of the probes are input to the processor **59**. The processor **59** is

5 configured to analyze the signals so as to determine an SPR parameter for the microspot. The processor is further configured to analyze the SPR parameter so as to monitor accumulation of the probe immobilized at each microspot. Signals **57** from regions of the probe surface that are not microspots are analyzed by the processor to correct and normalize signals from of the microspots.

10   After termination of the flow of the probe solutions in the microchannels, buffer or water is again made to flow through the microchannels **36** to eliminate unbound probe proteins.

The flow cell **34** now removed from the SPR surface and a second flow cell (not shown) having n microchannels is positioned on the SPR surface with a

15 microchannel overlying each of the n strip electrodes **33**. In the case that m=n, the flow cell **34** may also be used as the second flow cells by rotting it 90° from the orientation shown in Fig. 2 to an orientation (not shown) in which the microchannels **36** overlay the strip electrodes **33**. A solution containing the target is made to flow in each of the n microchannels. In this embodiment, the target is

20 present in the different microchannels at a different concentration. Thus, for each of the n target solutions, the target is presented to each of the m probe concentrations in the m microspots **58** located at the m crossover regions of the targets microchannel with the m probe regions. The signals **57** provided by the CCD **54** responsive to light from the light sources reflected from each of the n X

25 m microspots **58** during flow of the target molecules in the microchannels are input to the processor **59**. The processor **59** is configured to analyze these signals in order to monitor the binding of target to probe at each microspot. This allows collection of kinetic data on the binding of the target to the probe for kinetic analysis in a single binding assay, without the need to regenerate the surface at

30 any time. This is in contrast to prior art methods in which reactions are

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performed sequentially, each time with a different combination of probe and target concentrations which requires regeneration of the surface between successive binding reactions.

5   **Examples**

        Example 1

        A binding assay was carried out using the system **10** shown in Fig. 1. Anti-IL-4 antibody (αIL-4) was used as a probe in this experiment and was localized on

10   the SPR surface in each of six rectangular probe regions **42** (see Fig. 1), as explained above in the description of the system **10**. The probe regions were labeled (a) to (f). The density of the antibody, in *"response units"* (RU), in each of the 6 probe regions is given in Table 1.

15

Probe region	Target (αIl-4) concentration (pg/mm^2) (RU)
(a)	530
(b)	315
(c)	346
(d)	360
(e)	334
(f)	355

Table 1

        IL-4 was used as the target in this experiment was presented to the αIL-4 in each of five target regions **43** (see Fig. 1), as explained above. The target regions

20   were numbered 1 to 5. The concentration of IL-4 in each target region is given in Table 2.

Probe Localization Site	Target (IL-4) concentration (nM)
(1)	26.7
(2)	8.89
(3)	2.96
(4)	0.98
(5)	0.33

Table 2

The binding assay thus involved 30 binding reactions that were performed

5   simultaneously. Binding of IL-4 to αIL-4 in the 30 microspots was monitored
simultaneously as described above. The results of the binding are shown in Fig. 5.
Each graph in Fig. 5 shows binding of IL-4 to αIL-4 in the probe region indicated
in the graph. Each of the 5 curves in the graph shows the results of the binding of
IL-4 to αIL-4 in the microspot located at the intersection of the probe region of the

10   graph and the target region specified for each curve. At the times indicated by the
arrow in each graph, unbound IL-4 was rinsed away, and the dissociation of bound
IL-4 from αIl-4 in the 30 microspots was monitored simultaneously by the method
of the invention. The processor **63** was configured to analyze the curves in each
graph to obtain the association constant (Ka) and the dissociation constant (Kd) of

15   the binding of Il-4 to αIL-4 at the antibody concentration of the graph. The Ka and
Kd of each graph are shown in each of the graphs in Fig. 5. From these, the affinity
constant (KD) can be derived, as is known in the art.

Example 2

20   Binding between 6 antibody probes (αIgG1, αIgG2b, αIgA, αIgG2a and
αIgG3) to 5 antigen targets (IgG1, IgG1, IgG2a, IgG2b and IgG3) was studied
using the system **10** of Fig.1. The concentrations used of the probes and targets

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are given in Tables 3 and 4, respectively. The binding curves obtained are shown in Fig. 6 and the binding response of each of the 30 binding reactions is shown in Table 5.

5

10

Table 3

Probe	Probe region	Probe concentrations (pg/mm^2) (RU)
Anti mouse IgG2a	a	3410
Anti mouse IgG2b	b	4170
Anti mouse IgG1	c	3970
Anti mouse IgG3	d	3500
Anti mouse IgA	e	3770
Reference surface	f	-

Table- 4

15

Target	Target concentrations(μg/ml)
IgG1 (anti IL-2)	2.5
IgG1 (anti IL-4)	2.5
IgG2a	5

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IgG2b	5
IgG3	5
Mouse IgG	5

The binding responses to different antibody subclasses

(in response units)

5

Ligand Analyte	Anti mouse IgG2a	Anti mouse IgG2b	Anti mouse IgG1	Anti mouse IgG3	Anti mouse IgA
IgG1 (anti IL-2)	21	42	44	-	-
IgG1 (anti IL-4)	23	-	45	-	-
IgG2a	90	-	-	-	-
IgG2b	-	241	-	-	-
IgG3	-	-	-	97	-
IgG polyclonal	122	67	44	30	-

Table 5

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Example 3

The binding of five Cytochrome-P450 (CYP) enzyme probes (3A4, 2C19, 1A2, 2C9 and 2D6) with 6 different targets (Ketoconazole, Nifedipine, 15 Dextromethorphan, Diclofenac, Dulfaphenazole and Propranolol) was carried out using the system **10** of Fig. 1. The targets were presented at concentrations of

1,000, 500, 250, 125, 62.5, 31.25, 15.5, and 7.8 µM. The affinity constant, KD was determined for each reaction. The results are shown in Fig-7 and Table 6.

5   Table 6: Affinity constants (*KD* in [M]) determined for binding of various compounds to five CYP enzymes.

	CYP –P450				
	3A4	2C19	1A2	2C9	2D6
Ketoconazole	2.59E-05	5.21E-05	1.33E-03	7.65E-05	2.10E-04
Nifedipine	1.84E-03	2.24E-03	5.81E-02	1.42E-03	4.37E-03
Dexomethorphan	1.26E-02	7.90E-03	-	2.83E-02	6.04E-02
Diclofenac	4.47E-04	7.17E-04	1.42E-02	1.66E-04	6.81E-04
Sulfaphenazole	1.65E-01	1.14E-02	1.15E-03	2.06E-03	7.11E-02
Propranolol	7.53E-02	6.59E-03	8.73E-04	5.13E-03	5.22E-03

Example 4

Table 7 shows immobilization of Rabbit IgG and Goat IgG probes on 36
10   independent microspots prepared by the method shown in Fig. 3, using the system 10 of Fig. 1. Each probe region was sequentially activated and six alternate probes of Rabbit IgG and Goat IgG were adsorbed onto the activated probe region. This resulted in the immobilization of 36 alternate probes in the 36 microspots (6 in each surface region), as shown in Table 7.

15

20

Table 7

5

	1	2	3	4	5	6
1	Goat IgG 543 RU	Rabbit IgG 1640 RU	Goat IgG 963 RU	Rabbit IgG 1950 RU	Goat IgG 1050 RU	Rabbit IgG 1420 RU
2	Rabbit IgG 1620 RU	Goat IgG 1060 RU	Rabbit IgG 1800 RU	Goat IgG 1020 RU	Rabbit IgG 1320 RU	Goat IgG 1060 RU
3	Goat IgG 525 RU	Rabbit IgG 1870 RU	Goat IgG 1200 RU	Rabbit IgG 1960 RU	Goat IgG 1300 RU	Rabbit IgG 1430 RU
4	Rabbit IgG 1730 RU	Goat IgG 1300 RU	Rabbit IgG 2070 RU	Goat IgG 1240 RU	Rabbit IgG 1540 RU	Goat IgG 1360 RU
5	Goat IgG 608 RU	Rabbit IgG 1660 RU	Goat IgG 1200 RU	Rabbit IgG 2160 RU	Goat IgG 1340 RU	Rabbit IgG 1730 RU
6	Rabbit IgG 1680 RU	Goat IgG 1080 RU	Rabbit IgG 1910 RU	Goat IgG 1120 RU	Rabbit IgG 1530 RU	Goat IgG 1110 RU

Mouse anti-rabbit and mouse anti-goat antibody targets were then presented to the probe array. Table 8 shows the target binding responses. Each of the 36 independently selected probes in the probe array reacts with its
10   corresponding target allowing 36 different and independent interactions to be performed and monitored simultaneously (in a "checker board" pattern).

15

5

Table 8

1	2	3	4	5	6
Anti Rabbit (0 RU) Anti Goat (308 RU)	Anti Rabbit (348 RU) Anti Goat (5 RU)	Anti Rabbit (2 RU) Anti Goat (553 RU)	Anti Rabbit (431 RU) Anti Goat (0 RU)	Anti Rabbit (1 RU) (Anti Goat (584 RU)	Anti Rabbit (291 RU) Anti Goat (0 RU)
Anti Rabbit (354 RU) Anti Goat (0 RU)	Anti Rabbit (0 RU) Anti Goat (573 RU)	Anti Rabbit (415 RU) Anti Goat (0 RU)	Anti Rabbit (0 RU) Anti Goat (571 RU)	Anti Rabbit (262 RU) (Anti Goat (1 RU)	Anti Rabbit (0 RU) Anti Goat (579 RU)
Anti Rabbit (4 RU) Anti Goat (299 RU)	Anti Rabbit (402 RU) Anti Goat (4 RU)	Anti Rabbit (1 RU) Anti Goat (650 RU)	Anti Rabbit (435 RU) Anti Goat (1 RU)	Anti Rabbit (0 RU) (Anti Goat (687 RU)	Anti Rabbit (291 RU) Anti Goat (1 RU)
Anti Rabbit (362 RU) Anti Goat (1 RU)	Anti Rabbit (0 RU) Anti Goat (674 RU)	Anti Rabbit (480 RU) Anti Goat (0 RU)	Anti Rabbit (0 RU) Anti Goat (660 RU)	Anti Rabbit (309 RU) (Anti Goat (1 RU)	Anti Rabbit (0 RU) Anti Goat (704 RU)
Anti Rabbit (0 RU) Anti Goat (353 RU)	Anti Rabbit (355 RU) Anti Goat (0 RU)	Anti Rabbit (3 RU) Anti Goat (642 RU)	Anti Rabbit (475 RU) Anti Goat (0 RU)	Anti Rabbit (1 RU) (Anti Goat (708 RU)	Anti Rabbit (360 RU) Anti Goat (0 RU)
Anti Rabbit (358 RU)	Anti Rabbit (0 RU)	Anti Rabbit (435 RU)	Anti Rabbit (1 RU)	Anti Rabbit (300 RU)	Anti Rabbit (0 RU)

	Anti Goat	Anti Goat	Anti Goat	Anti Goat	Anti Goat	Anti Goat
	(4 RU)	(580 RU)	(2 RU)	(602 RU)	(2 RU)	(595 RU)